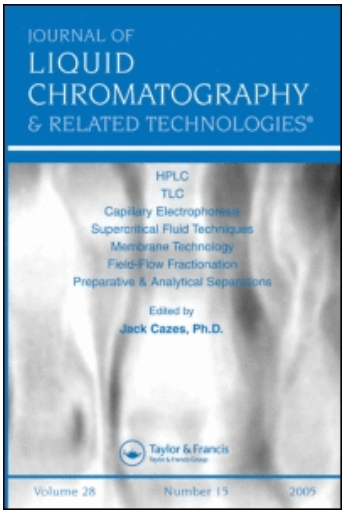

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High-Performance Liquid Chromatographic Determination of Lanatosides in *Digitalis Lutea* and *Digitalis Ambigua* Leaves

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF LANATOSIDES IN *DIGITALIS LUTEA* AND *DIGITALIS AMBIGUA* LEAVES

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ABSTRACT

An quantitative method for the determination of lanatoside A and lanatoside B in *Digitalis lutea* and *Digitalis ambigua* leaves by high-performance liquid chromatography (HPLC) is described. The extract of dry leaf powder with chloroform:ethanol (1:2, v/v) was submitted to Sep-Pak cartridges prior to HPLC analysis. HPLC was performed on an ODS column using methanol:water (2:1, v/v) for *Digitalis lutea* and a phenylsilyl bonded silica column with acetonitrile:water (5:8, v/v) for *Digitalis ambigua*. The effluent was monitored by ultraviolet (UV) absorption at 220 nm. The quantitation was carried out by the internal standard method. The present method is sufficiently sensitive and reproducible to assay lanatosides in *Digitalis* leaves.

INTRODUCTION

The cardiac glycosides prepared from *Digitalis* leaves are therapeutically important substances for the treatment of heart disease. For the determination of the glycosides in *Digitalis* leaves, thin-layer chromatography (TLC) has been shown to be a

useful method (1,2). However, HPLC seems to be more efficient for the analysis of the glycosides. The separation of various mixtures of pure cardiac glycosides has been achieved by the use of a normal-phase silica column (3-5) and a reversed-phase column (4,6). In addition, the usefulness of adapting gradient elution to the HPLC separation of the plant extract has been shown (7,8).

The genus *Digitalis* comprises about 25 species, of which *Digitalis lutea* and *Digitalis ambigua* (syn. *Digitalis grandiflora*) are known to contain lanatosides as primary glycosides. The published methods for the analysis of cardiac glycosides in *Digitalis lutea* and *Digitalis ambigua* have utilized paper chromatography (9,10), TLC (11), and HPLC employing gradient elution (12-14). In the previous paper of this serise, we reported the determination of purpurea glycosides in *Digitalis purpurea* leaves (15) and lanatosides in *Digitalis lanata* leaves (16), by means of HPLC. The present paper describes the convenient method for the simultaneous determination of lanatoside A and lanatoside B from the extract of *Digitalis lutea* and *Digitalis ambigua* leaves, which involves clean-up with Sep-Pak cartridges and subsequent separation by reversed-phase HPLC with isocratic elution.

MATERIALS AND METHODS

Chemicals

Lanatoside A and lanatoside B were purchased from E. Merck (Darmstadt, F.R.G.), and their chemical structures are given in Figure 1. 14α,15α-Epoxy-"β"-anhydrodesacetyllanatoside A, used as the internal standard, was synthesized in four steps from desacetyllanatoside A by the method adapted from Sawlewicz *et al.* (17). Desacetyllanatoside A was prepared from lanatoside A according to the procedure of Pekić and Miljković (18). All of these compounds were checked for homogeneity by TLC, and solvents were purified by redistillation prior to use.

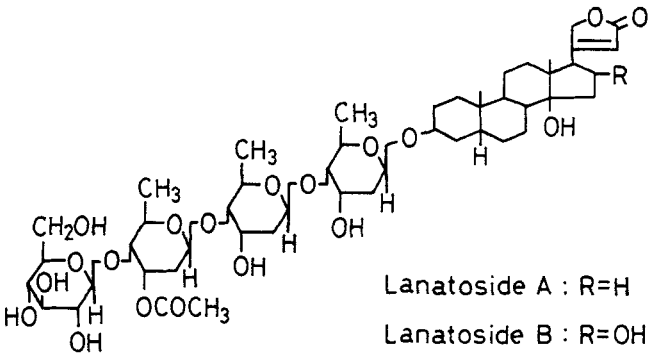

FIGURE 1. Chemical structures of the lanatosides investigated.

Chromatographic Apparatus

The HPLC system consisted of a JASCO 880-PU pump (Japan Spectroscopic, Tokyo, Japan), a Model KHP-UI-130 injector (Kyowa Seimitsu, Tokyo), a Model UV-8010 variable-wavelength detector (Tosoh, Tokyo), and a Chromatopac C-R3A data processor (Shimadzu, Kyoto, Japan). The Cosmosil $5C_{18}$ (5 μm, 150 x 4.6 mm I.D.)(Chemco Scientific, Osaka, Japan) and Cosmosil 5Ph (5 μm, 300 x 4.6 mm I.D.) columns were used at ambient temperature.

Preparation of *Digitalis* Leaf Powder

Leaves of *Digitalis lutea* L. and *Digitalis ambigua* Murr. were collected during the flowering stage in June at the Medicinal Plant Garden (Kanazawa, Japan) of Hokuriku University. These fresh leaves were immediately freeze-dried in a Neocool Model DC-55A apparatus (Yamato Scientific, Tokyo) and then dried using phosphorus pentoxide under reduced pressure at room temperature. The dried leaves were pulverized and sifted through a sieve of mesh width 500 μm. The leaf powder obtained was further dried using phosphorus pentoxide under reduced pressure for five days.

Sample Preparation for HPLC

Leaf powder (*ca.* 50 mg) of *Digitalis lutea* or *Digitalis ambigua* was accurately weighed and extracted with 25 ml of chloroform:ethanol (1:2, v/v) containing 14α,15α-epoxy-"β"-anhydrodesacetyllanatoside A (24.36 μg) as an internal standard. After ultrasonication for 1 hr in an ultrasonic cleaning bath, the extract was filtered and evaporated to dryness using a rotary evaporator. The residue was dissolved in 1 ml of ethyl acetate:ethanol:acetic acid (100:1:0.1, v/v) and subjected to the Sep-Pak silica cartridge (Waters, Milford, MA). Then 19 ml of ethyl acetate:ethanol:acetic acid (100:1:0.1, v/v) and 10 ml of ethyl acetate:ethanol:acetic acid (100:20:0.12, v/v) were successively passed through the cartridge. After evaporation of the latter fraction (10 ml) using a rotary evaporator, the resulting residue was dissolved in 1 ml of methanol:water:acetic acid (20:30:0.05, v/v) and loaded on the Sep-Pak C_{18} cartridge. After washing with 14 ml of methanol:water:acetic acid (20:30:0.05, v/v), lanatosides were eluted with 15 ml of methanol:water:acetic acid (20:10:0.03, v/v). The eluate was evaporated to dryness *in vacuo*. The material obtained was submitted to HPLC.

HPLC Determination

The HPLC determination of lanatosides in *Digitalis lutea* leaves was achieved by using a Cosmosil $5C_{18}$ column. The mobile phase used for the separation was methanol:water (2:1, v/v) and the flow rate was adjusted to 0.6 ml/min. The HPLC for *Digitalis ambigua* leaves was performed on a Cosmosil 5Ph column with acetonitrile:water (5:8, v/v) at the flow rate of 0.4 ml/min. The effluent was monitored by UV absorption at 220 nm. The extract pretreated above was dissolved in 0.5 ml of the mobile phase and a 10 μl volume of the sample solution was injected into the liquid chromatograph. Lanatoside A and lanatoside B in *Digitalis* plants were determined by the internal standard method. Calibration graphs were constructed by plotting the ratio of the peak area of lanatoside A or lanatoside B to the peak area of the internal

standard against the weight of each compound. The average peak areas from three chromatograms were used for the determination.

TLC Procedure

Normal- and reversed-phase TLC were performed on high-performance silica gel 60 F_{254} plates (5 x 10 cm, E. Merck) and KC_{18} F plates (5 x 10 cm, Whatman, Clifton, NJ), respectively. The plates were developed in glass chamber, checked by UV light around 254 nm, sprayed with concentrated sulfuric acid, and heated at 120 °C for 10 min.

RESULTS AND DISCUSSION

The HPLC determination of lanatosides in the leaves was carried out by the incorporation of an internal standard in order to improve the reproducibility on the clean-up procedure and the chromatographic run. Many compounds were investigated and 14α,15α-epoxy-"β"-anhydrodesacetyllanatoside A, which can be separated satisfactorily from lanatoside A and lanatoside B, was selected. The dried leaf powder was extracted with chloroform: ethanol (1:2, v/v) by ultrasonication. For the purpose of removing the many other plant materials, the extract was submitted to Sep-Pak cartridges packed with silica gel and ODS bonded silica gel prior to HPLC (Figure 2). The purified material was subjected to HPLC on a reversed-phase column. A detection wavelength of 220 nm was used, account being taken of the α,β-unsaturated lactone ring attached at the C-17 position of the steroid nucleus.

For the determination of lanatoside A and lanatoside B in *Digitalis lutea* leaves, the HPLC separation was performed on an ODS bonded silica column using methanol:water (2:1, v/v) as the mobile phase at a flow rate of 0.6 ml/min. Figure 3(a) shows the chromatogram of a standard mixture of lanatoside A, lanatoside B, and the internal standard. The separation is sufficiently good

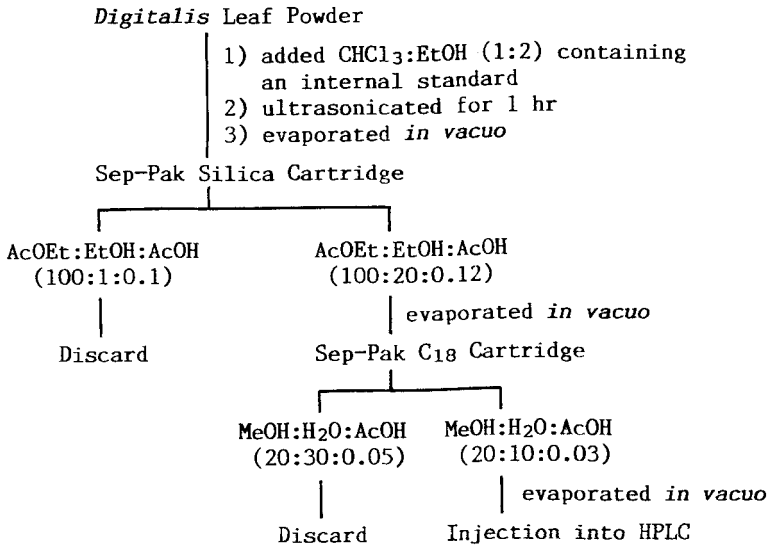

FIGURE 2. Procedure of the sample preparation for the HPLC determination of lanatosides in *Digitalis* leaves.

and reproducible to permit quantitative work. The representative chromatogram of the extract with the internal standard is given in Figure 3(b). From a chromatogram of the extract in the absence of the internal standard, the other substances present in the leaves were ascertained not to interfere with the peak of the internal standard. To make sure that the peaks of lanatoside A and lanatoside B were homogeneous, the eluate corresponding to each peak was collected and analyzed by TLC. Both normal-phase TLC (lanatoside A, R_f 0.48; lanatoside B, R_f 0.42) using chloroform:methanol:water (80:20:2.5, v/v) as developing solvent and reversed-phase TLC (lanatoside A, R_f 0.45; lanatoside B, R_f 0.55) using acetonitrile:0.5 *M* sodium chloride (10:13, v/v) indicated single components. For the HPLC quantitation, linear calibration graphs were prepared by plotting seven data points in the ranges of 10∿80 μg for lanatoside A and 5∿40 μg for lanatoside B. The regression equations and correlation coefficients (r) were deter-

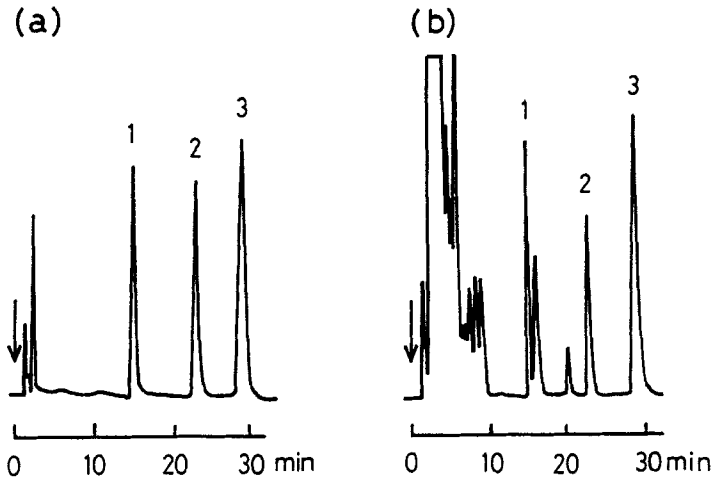

FIGURE 3. HPLC separations of lanatoside A, lanatoside B, and an internal standard for *Digitalis lutea* leaves. Peak identification: 1=lanatoside B; 2=14α,15α-epoxy-"β"-anhydrodesacetyllanatoside A; 3=lanatoside A. Chromatographic conditions: Cosmosil $5C_{18}$ column (150 x 4.6 mm I.D.); mobile phase, methanol:water (2:1, v/v); flow rate, 0.6 ml/min; UV detection at 220 nm; sample volume, 10 µl. Chromatogram(a): mixture of pure compounds. Chromatogram(b): extract of *Digitalis lutea* leaves with an internal standard.

mined as $y=0.0505x + 0.0231$ ($r=0.997$) for lanatoside A and $y=0.0472x - 0.0115$ ($r=0.998$) for lanatoside B, where y represents the peak area ratio of lanatosides to the internal standard and x the amount (µg) of lanatosides. The assay results obtained from ten dry leaf powder samples of *Digitalis lutea* are compiled in Table 1. The data indicate that the average contents of lanatoside A and lanatoside B per 100 mg of the leaf powder were 77.7 and 43.6 µg, respectively, with good reproducibility. The amount of lanatoside A in *Digitalis lutea* was 1.8 times higher than that of lanatoside B.

The determination of lanatosides in *Digitalis ambigua* leaves was also undertaken. When an ODS bonded silica column was used, the presence of co-extracted constituents of the leaves inter-

TABLE 1

Contents of Lanatoside A and Lanatoside B in *Digitalis lutea* Leaves Determined by the Present Method

Glycoside	Found* (μg)		Mean ± S.D. (μg)	C.V. (%)
Lanatoside A	81.3	78.5		
	77.1	73.2		
	74.8	77.6		
	77.9	75.6		
	77.7	83.3	77.7 ± 3.0	3.9
Lanatoside B	44.1	44.2		
	43.8	43.1		
	43.1	43.7		
	43.0	43.8		
	43.8	43.3	43.6 ± 0.4	0.9

*Values are the amount of lanatoside A or lanatoside B per 100 mg of a dry leaf powder sample.

fered with the peaks of lanatosides. For the complete HPLC separation of lanatosides in the leaves, a phenylsilyl bonded silica was employed as the stationary phase. Figure 4(a) depicts the chromatogram of a mixture of lanatoside A, lanatoside B, and the internal standard. These compounds were separated by using a solvent system consisting of acetonitrile:water (5:8, v/v) at a flow rate of 0.4 ml/min. The typical chromatogram of the extract after incorporation of the internal standard is illustrated in Figure 4(b). The purity of the peaks of lanatoside A and lanatoside B was also checked by both normal-phase TLC and reversed-phase TLC in the same manner as described above. The calibration graphs were obtained by plotting the peak area ratios (y) of lanatosides to the internal standard against the amount (x μg) of lanatosides. The regression equations were $y=0.0501x + 0.0080$ ($r=0.998$) for lanatoside A and $y=0.0462x - 0.0073$ ($r=0.998$) for lanatoside B. The quantitative analysis indicated that *Digitalis ambigua* leaves contained 62.6 μg of lanatoside A and 27.8 μg of lanatoside B per 100 mg of the dry leaf powder (Table 2). The content of lanatoside A in *Digitalis ambigua* leaves was about 2.3 times that of lanatoside B.

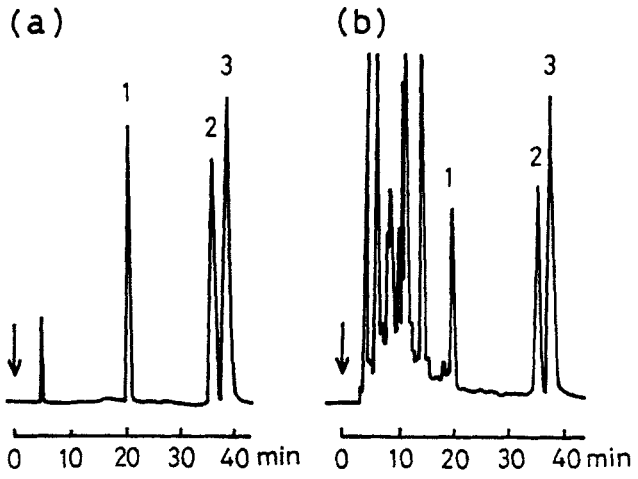

FIGURE 4. HPLC separations of lanatoside A, lanatoside B, and an internal standard for *Digitalis ambigua* leaves. Peak identification: 1=lanatoside B; 2=14α,15α-epoxy-"β"-anhydrodesacetyllanatoside A; 3=lanatoside A. Chromatographic conditions: Cosmosil 5Ph column (300 x 4.6 mm I.D.); mobile phase, acetonitrile:water (5:8, v/v); flow rate, 0.4 ml/min; UV detection at 220 nm; sample volume, 10 µl. Chromatogram(a): mixture of pure compounds. Chromatogram(b): extract of *Digitalis ambigua* leaves with an internal standard.

TABLE 2

Contents of Lanatoside A and Lanatoside B in *Digitalis ambigua* Leaves Determined by the Present Method

Glycoside	Found* (µg)		Mean ± S.D. (µg)	C.V. (%)
Lanatoside A	62.9	61.2		
	61.2	62.8		
	64.1	63.9		
	62.4	62.5		
	62.2	62.4	62.6 ± 1.0	1.6
Lanatoside B	30.3	26.5		
	28.5	25.4		
	27.0	29.2		
	28.6	27.7		
	29.3	25.6	27.8 ± 1.7	6.1

*Values are the amount of lanatoside A or lanatoside B per 100 mg of a dry leaf powder sample.

In the present study, the HPLC determination of lanatoside A and lanatoside B in *Digitalis lutea* and *Digitalis ambigua* leaves was achieved under an isocratic elution by the internal standard method. The values of lanatosides in *Digitalis lutea* were higher than those in *Digitalis ambigua*. The pretreatment procedure using Sep-Pak cartridges before the HPLC analysis was of great importance in the elimination of interfering peaks of the chromatogram. In conclusion, the proposed method is precise and selective for the determination of lanatosides in the leaves of *Digitalis lutea* and *Digitalis ambigua*. This technique can be useful for estimation of the quality of the leaves.

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